

## TOBACCO SMOKE HEMOGLOBIN ADDUCTS IN MATERNAL AND FETAL BLOOD

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**ABSTRACT** The maternal-fetal exchange of the potent tobacco related human carcinogen, 4-aminobiphenyl, was studied in women smokers during pregnancy. Maternal and fetal blood samples were classified as coming from nonsmokers ( $n=74$ ), individuals smoking less than 1 pack of cigarettes per day ( $n=16$ ), individuals smoking 1 pack of cigarettes per day ( $n=19$ ), individuals smoking 1-2 packs of cigarettes per day ( $n=19$ ), and individuals smoking greater than 2 packs of cigarettes per day ( $n=20$ ). 4-Aminobiphenyl was extracted from both maternal and fetal blood samples using organic extractions and the released amine was qualitatively and quantitatively characterized by analysis of the samples by gas chromatographic and mass spectrometric analysis. Increasing levels of 4-aminobiphenyl-hemoglobin adducts were found as the smoking status of the women increased ranging from  $144 \pm 22.2$  (<1 pack per day) to  $633 \pm 87.9$  (>2 packs per day). A corresponding increase in the presence of fetal 4-aminobiphenyl hemoglobin adducts was also detected ( $74.3 \pm 17.8$ ; <1 pack/day to  $319 \pm 50.5$ ; >2 packs/day).

**Keywords** Hemoglobin, Tobacco smoke, Biomarkers, Maternal, Fetal

### INTRODUCTION

Tobacco smoke is one of the most prevalent sources of *in utero* exposure to toxic substances. Evidence from clinical and laboratory studies suggests that exposure of the fetus to tobacco smoke carcinogens is highly probable, and that potential for tobacco smoke-induced human transplacental cancers exists and merits serious attention [1]. Recent studies have demonstrated that tobacco smoke toxins readily cross the

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placental membrane [2,3,4]. Additional studies have shown that tobacco smoke induces placental and fetal enzyme systems capable of bioactivation of procarcinogens to carcinogenic and mutagenic derivatives [5,6]. Maternal smoking has also been shown to be associated with DNA damage in the placenta [7] and exposure to tobacco smoke *in utero* may result in an increase risk of development of childhood and adult cancers [8,9,10]. In laboratory studies, tobacco smoke related carcinogens such as benzo(a)pyrene and the tobacco-specific nitrosamines readily cross the placental membrane and form adducts with placental DNA [11,12]. In addition, transplacental carcinogenesis occurs in laboratory animals exposed to cigarette smoke condensate, diethylnitrosamine, 3-methylcholanthrene, tobacco specific nitrosamines, and benzo(a)pyrene [13,14,15].

Advances in the quantitative analysis of covalent adducts have made it possible to study the association between tobacco smoke exposure and carcinogen induced DNA damage in fetal tissues. Everson *et al.*, using the  $^{32}\text{P}$  postlabeling assay, has recently detected numerous DNA adducts in human placental tissue obtained from smokers [7,16]. Shamsuddin and Gan [17] have shown that benzo(a)pyrene forms adducts in placental tissue. These adducts have been characterized as benzo(a)pyrene 7,8-diol-9,10-epoxide (BPDE)-DNA adducts in human placenta by using anti-BPDE DNA antibody and light microscope immunocytochemistry. Manchester, *et al.* [5] recently measured BPDE-DNA adducts in human placenta using  $^{32}\text{P}$ -postlabeling and immunoaffinity chromatography.

The formation of hemoglobin adducts with various environmental compounds has recently been proposed as a potential biomarker of exposure to carcinogenic compounds [18,19,20]. Furthermore, hemoglobin adducts appear to be surrogate biomarkers of genotoxic damage [20,21]. The formation of adducts with various electrophilic compounds such as ethylene oxide and benzo(a)pyrene indicate that hemoglobin may serve as a potential biomarker of exposure to these as well as additional tobacco smoke carcinogens [18,20,21,22,23].

Numerous aromatic amines, including 4-aminobiphenyl, have been detected in tobacco smoke [24]. Since some of these amines are potent human bladder carcinogens, such as 4-

adducts with various nitro compounds have been proposed as a mechanism for the carcinogenicity of these compounds. Nitro adducts appear to be capable of causing DNA damage [20,21]. The electrophilic nitro compounds such as 4-nitrophenol indicate that hemoglobin adducts formed from exposure to these as well as to nitroaromatics [18,20,21,22,23]. Hemoglobin adducts of 4-aminobiphenyl, have been reported [24]. Since some of these compounds are known carcinogens, such as 4-

In this study we investigated the relationship between maternal smoking and 4-aminobiphenyl hemoglobin adduct levels in both maternal and fetal blood. 4-Aminobiphenyl, a tobacco related aromatic amine, is known to be a potent bladder carcinogen present in mainstream and sidestream smoke [24]. 4-Aminobiphenyl hemoglobin adducts may be useful as a biomarker of genotoxic damage in the fetus. The presence of significantly elevated levels of a potent tobacco-smoke carcinogen in the hemoglobin of maternal and fetal blood samples demonstrates the importance of studying the maternal - fetal exchange of carcinogens during pregnancy. In addition, the present study confirms, with a larger sample size, the previous series of investigations by Coghill, *et al.* [22], in which hemoglobin adducts with 4-aminobiphenyl in women smokers were analyzed.

4-Aminobiphenyl and 4-F-aminobiphenyl were purchased respectively from Fluka Chemika-Biochemika, Ronkonkoma, New York, and Sigma-Aldrich Chemical Co., Milwaukee, WI. All aqueous solutions were prepared with distilled deionized water. Trimethylamine in hexane was prepared by adding 1 g trimethylamine hydrochloride (Fluka Chem.-Biochem.) to 2 ml water, neutralizing the solution with NaOH and extracting with 10 ml hexane. The internal standard, 4'-F-aminobiphenyl was recrystallized from dichloromethane/hexane and used to prepare a stock solution of 25 ng/ml in 0.1 N HCl which was stored at 4°C. Pentafluoropropionic anhydride (PFPA) was purchased from Fluka. All the chemicals and reagents were of the highest grade commercially available.

Blood samples were obtained from Norton's Hospital and the University of Louisville Hospital. Women participating in the study were assessed as to their recent smoking habits via questionnaire and assessment by immunoassay (Abbott

Laboratories, Abbott Park, IL) of urine and serum cotinine levels. Maternal blood samples (10 ml) were collected into heparinized vacutainers from smoking and nonsmoking mothers during admission for labor and delivery. Fetal blood samples (5 ml) were collected from the umbilical vein into heparinized tubes immediately after delivery. Individuals were classified as to their smoking status and were divided into 5 groups. Nonsmokers ( $n=74$ ), less than 1 pack per day smokers ( $n=16$ ), 1 pack per day smokers ( $n=19$ ), 1-2 pack per day smokers ( $n=19$ ), and greater than 2 packs per day smokers ( $n=20$ ) were included in the study. Paired maternal and fetal blood samples were obtained from all individuals in the study.

#### Analysis of samples

Hemoglobin - 4-aminobiphenyl adducts were processed using the method of Bryant *et al.* [19] with modifications. Maternal and fetal blood samples were centrifuged at  $3,000 \times g$  to generate packed red blood cells. After removal of serum, the red cells were washed 3 times with 0.9% saline and lysed by the addition of 15 ml ice cold deionized water and 2 ml toluene with vigorous shaking. After 15 minutes, the lysate was removed by centrifugation at  $10,000 \times g$  for 20 minutes to remove cellular debris. The hemoglobin solution was transferred to dialysis tubing and dialyzed for 24 hours at  $4^\circ\text{C}$  against 2 changes of distilled, deionized water (2 liter). Hemoglobin concentrations were determined by measurement of the absorbance at 415 nm (oxyhemoglobin, extinction coefficient  $125 \text{ mM}^{-1}$ ). Samples were divided into aliquots (3-5 ml each) to allow for reproducibility of analysis and stored at  $-20^\circ\text{C}$  until analysis by gas chromatography and mass spectrometry.

#### Extraction of 4-aminobiphenyl hemoglobin adducts in maternal and fetal blood

Prior to extraction of the hemoglobin samples for gas chromatographic / mass spectrometric analysis of 4-aminobiphenyl, the hemoglobin samples (3 ml) were spiked by the addition of 400 pg of the internal standard 4'-F-aminobiphenyl. After spiking the hemoglobin sample, the hemoglobin solution was made 0.1 M in NaOH and incubated for 2 hours at room temperature. The hydrolysate was extracted twice with 15 ml of methylene chloride and the resulting emulsion broken by freezing and thawing the sample. The

urine and serum cotinine (0 ml) were collected into 10 ml and nonsmoking mothers (n=16). Fetal blood samples (5 ml) were collected from the umbilical vein into heparinized tubes. Mothers were classified as to their smoking status into 5 groups. Nonsmokers (n=16), 1 pack per day smokers (n=19), and 2 packs per day smokers (n=20) were included in the study. Fetal blood samples were analyzed.

Maternal and fetal blood samples were processed using the following modifications. Maternal blood was centrifuged at 3,000 x g to separate plasma from cells. After removal of serum, the cells were washed with 0.9% saline and lysed by adding 2 ml of distilled water and 2 ml of toluene. After 10 minutes, the lysate was centrifuged at 3,000 x g for 20 minutes to separate the cell debris. The supernatant solution was transferred to a clean vial and stored at 4°C for 24 hours. Hemoglobin was extracted by measurement of the optical density of the lysate at 415 nm. Aliquots (3-5 ml each) were stored at -20°C until analyzed by mass spectrometry.

**Hemoglobin adducts in maternal and fetal blood.** Hemoglobin samples for gas chromatographic analysis of 4-aminobiphenyl hemoglobin adducts (3 ml) were spiked by adding an internal standard 4-F-aminobiphenyl hemoglobin sample, the concentration of which was 1 M in NaOH and incubated at 37°C for 24 hours. The hydrolysate was extracted with 2 ml of hydrochloric acid and the resulting supernatant was analyzed by mass spectrometry. The

extracts were treated with 10 µl trimethylamine in hexane and derivatized by the addition of 5 µl pentafluoropropionic anhydride (PFPA) and the resulting derivatized products were evaporated under nitrogen. The residue was dissolved in 20 µl hexane, and 3 µl injected into the GC/MS for analysis.

#### Gas chromatographic and mass spectrometric analysis

Gas chromatographic and mass spectral analysis of the hemoglobin samples was carried out on a Hewlett-Packard 5890 Series II gas chromatograph (GC) connected to a 5971A mass selective detector. The GC oven was fitted with a DB-Wax 20 m capillary column (0.18 mm internal diameter, 0.3 µm film thickness) operating under the following parameters: 100°C initial temperature for 1 minute, ramp rate 20°C/min up to 240°C, held for 15 minutes (total analysis time = 23 minutes), injector 200°C, detector (MS) 180°C; inlet pressure of the carrier gas (helium) 3.0 psig. Single ion monitoring was accomplished by detecting the 4-aminobiphenyl-PFP ( $m/z$  315) and 4-F-aminobiphenyl-PFP ( $m/z$  = 333) derivatives. Data analysis was performed on a Hewlett-Packard Vectra QS/20 computer using the HP Chemstation software, version G1034C. Integrated peak areas of 4-aminobiphenyl and derivatives were used to calculate concentrations of 4-aminobiphenyl in the hemoglobin samples.

#### **RESULTS**

Seventy-four nonsmokers and seventy-four smokers were enrolled in the study. Maternal - fetal paired blood samples were obtained from all individuals enrolled in the study. 4-Aminobiphenyl hemoglobin adducts were detected in all maternal and fetal blood samples. Smokers were subdivided into 4 groups consisting of less than 1 pack/day smokers (n=16), 1 pack/day smokers (n=19), 1-2 packs per day smokers (n=19), and greater than 2 packs per day smokers (n=20).

The concentration of 4-aminobiphenyl - hemoglobin adducts in maternal blood samples was found to be significantly higher in smokers (mean,  $387 \pm 193$ ) when compared to nonsmokers (mean,  $18.3 \pm 12.7$ ). Additionally, the adduct level detected in cord blood samples of fetuses from smoking mothers (mean,  $184 \pm 99.7$ ) was also significantly higher than the concentration

of adduct detected in the cord blood from nonsmokers (mean,  $8.88 \pm 5.80$ ). A comparison of the adduct ratios between maternal and fetal 4-aminobiphenyl hemoglobin adducts in smokers and nonsmokers is shown in figure 1. In paired samples from nonsmokers, the ratio of maternal to fetal adduct was found to be  $2.18 \pm 0.77$ . In our smoker population, this ratio between maternal adduct level and fetal adduct level was found to be  $2.03 \pm 0.32$ . This ratio between maternal and fetal adducts in smokers and nonsmokers corresponds with ratios reported by Coghlin, *et al.* [22].

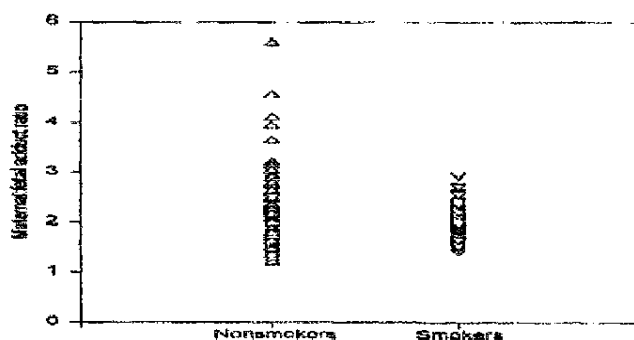
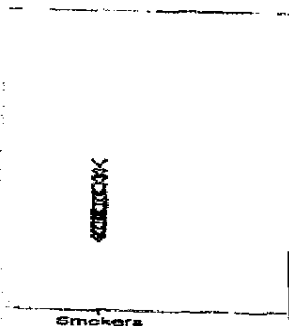


FIGURE 1: Ratio of maternal to fetal 4-aminobiphenyl hemoglobin adduct concentration in nonsmokers ( $n=74$ ) and smokers ( $n=74$ ).

The analysis of maternal 4-aminobiphenyl hemoglobin adducts is shown in figure 2. Nonsmokers had a background level of adduct of  $18.3 \pm 12.7$  pg 4-aminobiphenyl / g hemoglobin. As smoking status of the women increased, a corresponding increase in the detection of 4-aminobiphenyl hemoglobin adduct (from  $144 \pm 22.2$  to  $633 \pm 87.9$  pg 4-aminobiphenyl / g hemoglobin) was detected (Figure 2). This increasing level of adduct corresponds to increased exposure of 4-aminobiphenyl through tobacco smoke exposure. Similarly, 4-aminobiphenyl hemoglobin adduct in fetal blood was determined as described. A similar, but reduced level of adduct

from nonsmokers (mean, 8.88  $\pm$  5.8 pg 4-aminobiphenyl / g hemoglobin adducts in maternal blood in figure 1. In paired comparison of maternal to fetal adduct levels in smoker population, this ratio was found to be 1.0. Fetal adduct level was found to be 1.0 times maternal and fetal adducts with ratios reported by

was determined in fetal blood from nonsmoking mothers (Figure 3). Fetal cord blood obtained from nonsmokers had a mean adduct level of 8.88  $\pm$  5.8 pg 4-aminobiphenyl / g hemoglobin. This level was found to increase from 74.3  $\pm$  17.8 pg 4-aminobiphenyl / g hemoglobin to 319  $\pm$  50.5 pg 4-aminobiphenyl / g hemoglobin as the smoking status of the mothers increased from less than one pack of cigarettes per day to greater than 2 packs of cigarettes per day. A comparison of the levels of 4-aminobiphenyl adduct detected in maternal and fetal blood samples is shown in table 1.



4-aminobiphenyl hemoglobin adducts (n=74) and smokers

4-aminobiphenyl hemoglobin adducts in maternal blood from nonsmokers and smokers. Nonsmokers had a background level of 8.88  $\pm$  5.8 pg 4-aminobiphenyl / g hemoglobin. As the women increased, a level of 4-aminobiphenyl adducts increased from 74.3  $\pm$  17.8 pg 4-aminobiphenyl / g hemoglobin to 319  $\pm$  50.5 pg 4-aminobiphenyl / g hemoglobin. This increase was due to increased exposure to tobacco smoke. Similarly, 4-aminobiphenyl adducts in fetal blood were found to be at a reduced level of adduct

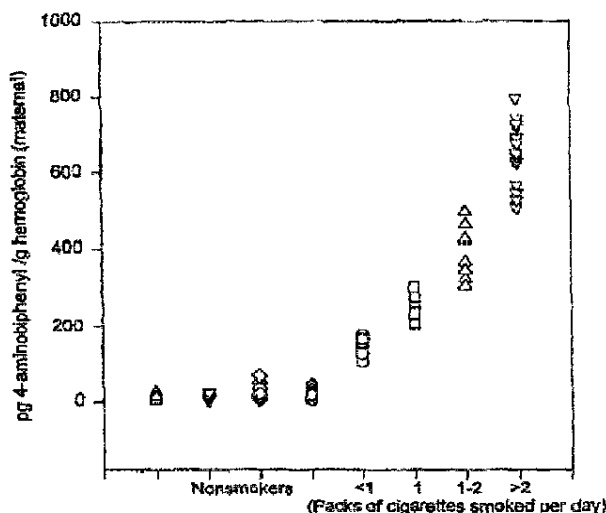


FIGURE 2: Comparison of 4-aminobiphenyl hemoglobin adducts in maternal blood from nonsmokers and smokers.

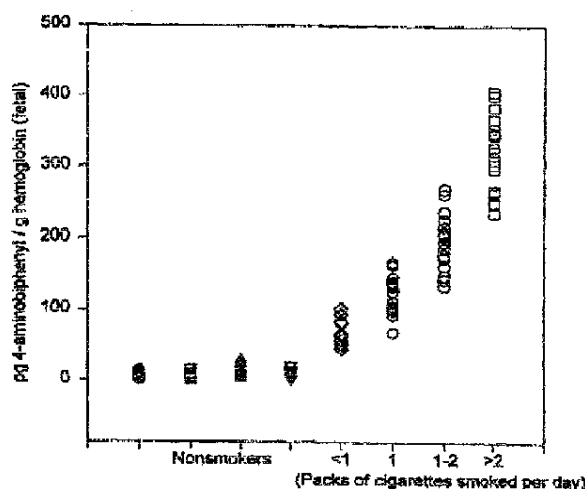
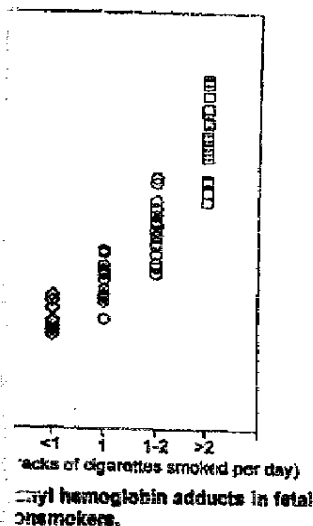


FIGURE 3: Comparison of 4-aminobiphenyl hemoglobin adducts in fetal blood obtained from smokers and nonsmokers.

In order to characterize the overall relationship between maternal exposure to the carcinogen 4-aminobiphenyl and fetal exposure, we carried out linear regressions on the data from our populations using maternal 4-aminobiphenyl hemoglobin adduct as the independent variable and fetal adduct levels as the dependent variable. When all of the samples were pooled for analysis (Figure 4), a significant correlation between maternal and fetal exposures to 4-aminobiphenyl was detected in our sample population ( $r^2 = 0.97$ ,  $p < 0.001$ ). In order to determine to what extent the correlation is influenced by smoking status of the individuals, as well as nonsmokers, separate regression analyses were carried out with each group of smokers. The results for the less than 1 pack per day smokers (Figure 5) yielded a correlation of  $r^2 = 0.335$ , 1 pack/day a correlation of  $r^2 = 0.488$  (Figure 6), 1-2 packs/day a correlation of  $r^2 = 0.585$  (Figure 7), and greater than 2 packs/day, a correlation between maternal 4-aminobiphenyl hemoglobin adducts and fetal adducts of  $r^2 = 0.406$  (Figure 8).



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## DISCUSSION

This study demonstrates that the potent tobacco related carcinogen, 4-aminobiphenyl, or its active metabolite, N-hydroxy-4-aminobiphenyl, crosses the human placenta and binds to fetal hemoglobin. All fetal blood samples tested revealed detectable amounts of 4-aminobiphenyl hemoglobin adducts. Carcinogen hemoglobin adduct levels in the fetuses of smoking mothers were significantly higher than in the levels measured in the fetuses of non-smoking mothers. The data presented represents an extension of the work of Coghlin, *et al.* [22], in that a larger sample population was used and a further classification of smoker status was obtained. A consistent observation was the apparent 1.5 - 2.2 fold reduction in the formation of fetal hemoglobin aminobiphenyl adducts when compared to matched maternal samples.

## 4-AMINOBIPHENYL HEMOGLOBIN ADDUCTS

Smoking Status	pg 4-ABP / g Hb (maternal) (mean $\pm$ SD)	pg 4-ABP / g Hb (fetal) (mean $\pm$ SD)
nonsmokers: (n=74)	16.35 $\pm$ 12.72	8.08 $\pm$ 5.80
smokers: ( $<1$ pack/day; n=16)	144.53 $\pm$ 22.22	74.36 $\pm$ 17.87
smokers: (1 pack/day; n=19)	250.19 $\pm$ 33.16	123.31 $\pm$ 26.71
smokers: (1-2 packs/ day; n=19)	394.04 $\pm$ 64.43	196.45 $\pm$ 40.82
smokers: ( $>2$ packs/ day; n=20)	633.01 $\pm$ 87.96	319.17 $\pm$ 60.52

TABLE 1: Comparison of 4-aminobiphenyl hemoglobin adduct levels in smokers and nonsmokers

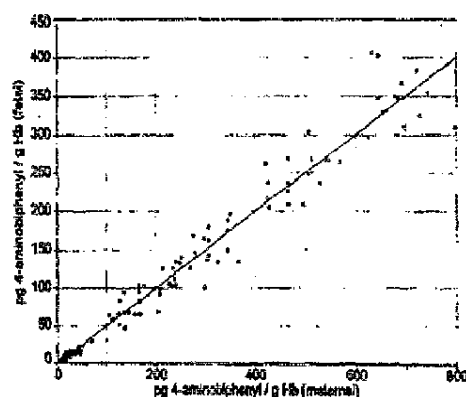


FIGURE 4: Linear regression analysis of maternal 4-aminobiphenyl hemoglobin adducts and fetal 4-aminobiphenyl hemoglobin adducts in the total study ( $n=148$ ).

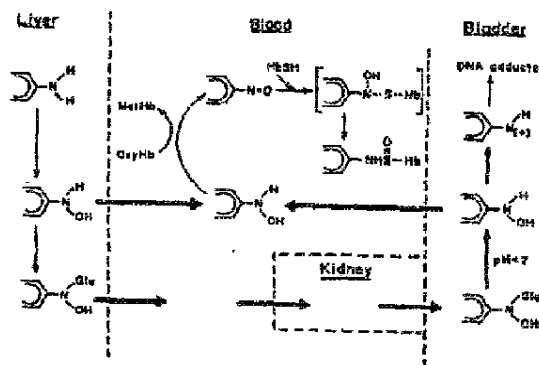
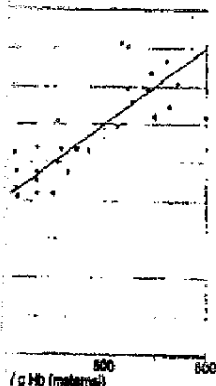
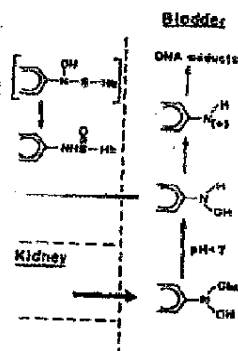


FIGURE 9: Scheme for the metabolism of the tobacco smoke carcinogen 4-aminobiphenyl and subsequent formation of hemoglobin and DNA adducts



maternal 4-aminobiphenyl  
biphenyl hemoglobin adducts  
=148).



olism of the tobacco smoke  
subsequent formation of  
DNA adducts

The approximate 2 fold difference in maternal and fetal levels of adduct observed in our study is consistent with animal studies of 4-aminobiphenyl hemoglobin transplacental transport. Lu *et al.* [11] found detectable levels of 4-aminobiphenyl DNA adducts in all fetal tissues following maternal dosing in laboratory rats and fetal levels were generally lower than maternal levels. Possible explanations for the lower fetal levels observed in our study include (1) an immaturity of fetal enzyme activating systems, (2) placental trapping of active metabolites, (3) carcinogen inactivation catalyzed by tobacco smoke induced placental enzymes, and (4) increase rate of degradation of 4-aminobiphenyl-fetal hemoglobin adducts. Studies have shown that fetal red blood cells turns over at a significantly faster rate (lifetime = 65 days) compared to maternal hemoglobin (lifetime 120 days). Therefore, if exposure to tobacco smoke decreased during the third trimester, relative lower levels of carcinogen hemoglobin adducts may be present in the fetal blood samples obtained at delivery.

The significantly elevated levels of 4-aminobiphenyl hemoglobin adducts in cord blood samples from smokers raises concerns regarding the potential for transplacental carcinogenesis. Although fetal levels in our study are consistently lower than maternal levels, studies of transplacental carcinogenesis in laboratory animals have shown that lower levels of carcinogens may initiate carcinogenesis when exposure occurs *in utero*. Administration of 60 mg ethylnitrosourea per kilogram body weight to pregnant rats initiates 50 times as many tumors in offspring as dose the same dose in adults [25]. In addition, the observation that enzyme systems are generally activated earlier in human fetuses than in laboratory animals supports the possibility that activated tobacco smoke carcinogens may be present in fetal tissues during cell proliferation and differentiation [26]. Various biomarkers of genotoxic damage have been proposed and carcinogen hemoglobin adducts have been shown to be accurate dosimeters of DNA adduct formation in adult humans and laboratory animals [27,28,29]. In the human fetus, however, DNA repair enzyme activity is twofold to fivefold lower than in the adult [30]. It is possible that DNA repair activity in the fetus

occurs at a slower rate and that DNA damage in the fetus is even greater than indicated by carcinogen hemoglobin adducts.

Several epidemiological studies have been conducted to look for a relationship between childhood and adult cancers and *in utero* exposure to tobacco smoke carcinogens. Stjernfeldt [8] reported a dose response relationship between number of cigarettes smoked per day during pregnancy and cancer risk in offspring. The risk is doubled for non-Hodgkin's lymphoma, acute lymphoblastic leukemia, and Wilm's tumor. In a large prospective study, Neutel and Buck [9] found a nearly doubled incidence of leukemia in the offspring of mothers who smoked during pregnancy. Sandler [10] reported an increased adult risk for hematopoietic malignancies related to gestational exposure to tobacco smoke. Significantly increased relative risk was found for Hodgkin's disease, non-Hodgkin's lymphoma, and acute leukemia. In a recent study, Janerich, *et al.* [31] reported that 17% of lung cancer among nonsmokers can be attributable to high levels of exposure to tobacco smoke during childhood and adolescence. In addition, *in utero* exposure may occur during a time of potentially increased vulnerability secondary to the rapid cell proliferation and differentiation in the developing fetus. In support of this hypothesis, Kauffman [32] demonstrated a close correlation between the number of proliferating epithelial cells and the number of tumors induced transplacentally by ethylnitrosourea at different gestational ages.

Butler *et al.* [33] found a 44-fold variation in rates of 4-aminobiphenyl N-oxidation in 22 liver microsome preparations, and Cartwright, *et al.* [34] demonstrated a greater than 10 fold person to person variation in the activity of several enzymes involved with benzo(a)pyrene metabolism. These findings may help explain the various individual levels of hemoglobin 4-aminobiphenyl adduct found in maternal and fetal blood. Vineis *et al.* [35] observed that levels of 4-aminobiphenyl hemoglobin adducts were higher in research subjects with genetically determined slow acetylation rates. In related studies, Manchester and Jacoby [36] observed substantial overlap and variability of placental monooxygenase activity in research subjects within the same smoke exposure groups.

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In our study, and in the work of Coghlin et al. [22], 4-aminobiphenyl hemoglobin adducts were detected in maternal

and fetal blood samples obtained from smoking mothers and non-smoking mothers during pregnancy. The presence of a detectable adduct level in nonsmokers suggests that there may be sources of human exposure to 4-aminobiphenyl other than cigarette smoking. Since our nonsmoker population group was found not to be exposed to passive smoke, we must assume that a dietary or ambient concentration of 4-aminobiphenyl is accounting for this small level of adduct in the blood of these individuals. Dietary contamination, such as the cooking of meats, which produces a number of heterocyclic amines, may contribute to the levels of 4-aminobiphenyl found in our population groups.

4-Aminobiphenyl hemoglobin adducts are believed to be formed *in vivo* through a series of reactions illustrated in Figure 9 occurring in either the liver or blood. The hydroxylamine, formed in the liver in a cytochrome P-450 mediated oxidation reaction, undergoes a subsequent cooxidation with oxyhemoglobin to yield N-nitrosobiphenyl and methemoglobin. The resulting nitrosoarene can either be converted back to the hydroxylamine or can react with suitable nucleophilic targets, such as cysteine, in hemoglobin forming covalent adducts. In summary, this study confirms transplacental passage of a potent tobacco related human carcinogen, 4-aminobiphenyl. The presence of significantly elevated levels of 4-aminobiphenyl hemoglobin adducts in the blood of fetuses from smoking mothers suggests that maternal smoking during pregnancy may increase carcinogen induced DNA damage in fetal tissues and may, therefore, be associated with increased risk of developing childhood and adult cancers. Future studies will investigate the formation of maternal - fetal hemoglobin adducts with various tobacco smoke carcinogens as well as other environmental carcinogens.

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